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Influence of Breed Size, Age, Fecal Quality, and Enteropathogen Shedding on Fecal Calprotectin and Immunoglobulin A Concentrations in Puppies During the Weaning Period

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Background: Fecal calprotectin and immunoglobulin A (IgA) are markers of intestinal inflammation and immunity in adult dogs.

Hypothesis: Fecal calprotectin and IgA concentrations in puppies are not influenced by fecal moisture in puppies but by enteropathogen shedding.

Animals: Three hundred and twenty-four puppies.

Methods: Fecal consistency was assessed by gross examination. Fecal moisture was evaluated before and after lyophilization. Canine parvovirus and coronavirus were detected in feces by qPCR and qRT-PCR respectively. *Giardia intestinalis* antigen was quantified by ELISA. The standard McMaster flotation technique was used to detect eggs and oocysts in feces. Fecal calprotectin and IgA concentrations were quantified by in-house radioimmunoassays.

Results: For each marker (IgA and calprotectin), a strong positive correlation was observed between concentration in fresh feces and concentration in fecal dry matter. 75.6% of the puppies were found to be infected by at ≥1 of the enteropathogens evaluated. Fecal calprotectin concentration was significantly influenced by age ($P = .001$), with higher concentrations in younger puppies, but not by viral ($P = .863$) or parasitic infection ($P = .791$). Fecal IgA concentration was significantly influenced by enteropathogen shedding ($P = .01$), with a lower fecal IgA concentration in puppies shedding at ≥1 enteropathogen compared to puppies without any enteropathogen shedding, but not by age.

Conclusions: Fecal calprotectin and IgA are of no diagnostic value to detect presence of enteropathogens in clinically healthy puppies or puppies with abnormal feces, but could help to better understand the maturation of digestive tract.

Key words: Age; Calprotectin; Digestive; Dog; Enteropathogens; Immunoglobulin A.

In dogs, gastrointestinal and hepatic diseases are the third most frequent problem reported by owners in United States and Australia.[1] In the United Kingdom, a survey based on client questionnaires reported that up to 15% of dogs experienced mild diarrhea over a 2-week period.[2] Another study observed that, among sick animals presented for veterinary consultation, 7% of visits were related to diarrhea.[3] Diarrhea is even more frequent in young dogs <6 months of age than in adult dogs, with 25% of puppies having abnormal feces during the weaning period.[4,5] Diarrhea during this period is a major problem as it can decrease daily weight gain and increase the risk of death.[6] As in other species, diarrhea is multifactorial, involving factors intrinsic to the dog (eg, breed size and age), nutritional factors (eg, diet change without transition, food type and quality), infectious diseases, and also lifestyle and environmental stressors.[5–11] Major enteropathogens associated with diarrhea in weanling puppies are canine parvovirus type 2 (CPV2),[12] the *Cystoisospora ohioensis*-complex, *Cystoisospora canis*,[13] and *Giardia duodenalis*.[14] Co-infections by these enteropathogens are frequently reported in puppies.[4,5] Several diagnostic tests (such as PCR, antigen testing by ELISA, and fecal examination) must be combined for accurate diagnosis in cases of diarrhea in weanling puppies. However, the use of such routine testing can be difficult and expensive.

In young children with gastroenteritis, several fecal markers are used to assess shedding of enteropathogens excreted, and to evaluate intestinal inflammation or local immunity.[15] Calprotectin and immunoglobulin A

Abbreviations:

CCECAI	canine chronic enteropathy clinical activity index
IgA	immunoglobulin A

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The work was done in various French breeding kennels.

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(IgA) are 2 of these markers. Calprotectin is a heterodimeric protein complex mainly present in neutrophils, monocytes, and reactive macrophages. In humans, fecal calprotectin concentrations were reported to be increased in patients with Crohn's disease or ulcerative colitis compared to healthy controls.[16,17] Moreover, they also correlated with disease severity as quantified by endoscopy and histologic examination of biopsy specimens.[18] In adult dogs with chronic diarrhea, significantly higher serum and fecal calprotectin concentrations have been reported compared to healthy dogs.[19,20] Sensitivity and specificity of fecal calprotectin for discriminating adult dogs with severe chronic diarrhea (Canine Chronic Enteropathy Clinical Activity Index [CCECAI] $\geq$ 12) from dogs with mild to moderate clinical signs (CCECAI < 12) were 53 and 92%, respectively, when a cut-off value of 49 μg/g was used.[19] Secretory IgA is the predominant immunoglobulin subtype present in secretions, protecting mucosal surfaces from infectious agents. Therefore, fecal IgA concentration may serve as a marker of mucosal immunity.[21] In dogs, fecal secretory IgA concentrations previously have been used to evaluate intestinal immunity.[22,23]

For interpretation of fecal IgA and calprotectin concentrations, how they are affected by physiological factors such as breed size or age must be taken into account. The canine species is characterized by large interbreed variations, primarily by stature. The digestive physiology of dogs is also known to differ slightly according to breed size. In large breed dogs, such as German shepherds or Great Danes, fecal moisture content is higher, soft stools are more frequent and the number of defecations is higher than in small breed dogs.[7,10,11,24] This difference may be a result of lower mineral absorption, higher fermentative activity reflecting higher intestinal permeability and a longer transit time, or both.[25–30] The same variation has been described in puppies, with large breed puppies having feces of lower consistency compared to small breed puppies.[6] Age also affects concentrations of markers. Indeed, lower fecal IgA concentrations were described in puppies <6 months of age compared to adult dogs.[31,32] In humans, an effect of age on fecal calprotectin concentration has been described with higher concentrations being observed in healthy children compared to healthy adults.[33] The same variation has been described between healthy puppies and healthy adults, but these results were obtained from dogs housed in the same breeding kennel.[32] Therefore, the aims of our study were to determine if calprotectin and IgA are influenced by fecal moisture (Study 1) and to evaluate if these fecal markers can be useful to detect infection by an enteropathogen (virus, parasite, or both) in puppies, taking into account the effect of 2 potential biases, age and breed size (Study 2).

Materials and Methods

The protocols of both studies were reviewed and approved by Royal Canin Internal Ethics Committee.

Study 1: Relationship Among Fecal Moisture Content, Fecal Quality and Fecal IgA and Calprotectin Concentrations

A total of 70 purebred puppies from 18 litters from 10 different breeding kennels were included. Each puppy was identified by a colored collar and its age and breed were recorded. Depending on the mean adult body weight of their respective breed, puppies were categorized into 2 groups (small breed if the mean adult body weight was <25 kg; otherwise large breed). For each puppy, fecal consistency was evaluated by a single operator using a 13-point scale, based on the texture and shape of the feces (from liquid to hard and dry).[6] Fresh feces were collected and weighed for each dog. If the stool volume defecated was sufficient ($\geq$15 g), stools were separated into 3 aliquots, 1 for fecal moisture evaluation and 2 for measurement of fecal calprotectin and IgA concentrations. Water content of the stools was determined by weighing feces before and after lyophilization.[30] Calprotectin and IgA were quantified by in-house radioimmunoassays after extraction, as previously described.[22,32,34] All samples were analyzed using the same batch of tracer and reagents. To correct for fecal moisture, results for each marker were expressed as concentrations in fresh feces and also normalized to dry matter.

Study 2: Relationship Between Fecal Markers and Enteropathogen Shedding

A total of 254 purebred puppies from 64 litters from 33 different French breeding kennels were included. Puppies vaccinated within the preceding 10 days before the visit and puppies with clinical signs of weakness, dehydration, or anorexia were not included in the study. However, puppies with an abnormal fecal quality were included in the study. Each puppy was identified by a colored collar and its age and breed were recorded. Depending on the mean adult body weight of their respective breed, puppies were categorized into small breed size or large breed size as described above. For each puppy, fecal consistency was evaluated by a single operator using a 13-point scale as previously described.[6] Based on growth rate deterioration, thresholds for abnormal feces in puppies were previously validated and appeared to vary with breed stature and age.[6] Briefly, feces with a score $\leq$5 were classified as abnormal for large breed puppies regardless of age. For small breed puppies, fecal scores $\leq$6 and $\leq$7 were classified as abnormal for 4–5 week old puppies and for older puppies between 6 and 8 weeks old respectively.

After collection, fecal samples were separated into 3 aliquots: 5 g of fresh feces were stored at +4°C for fecal examination and the other 2 samples were frozen at −20°C for *Giardia intestinalis* antigen quantification and measurement of fecal calprotectin and IgA concentrations respectively.

A rectal swab (medical dry swab, cotton tip diameter 2 mm[a]) was collected from each puppy immediately after stool collection for detection of CPV2 and canine coronavirus (CCV). The swabs were stored at −20°C until DNA extraction.

Fecal examination was performed by the standard McMaster flotation technique using a saturated magnesium sulfate solution (density: 1.28 g/mL).[35] All eggs and oocysts were identified according to their morphological characteristics under light microscopy by a single operator.[36,37] Copro-antigens of *G. intestinalis* were quantified by ELISA[b] in 100 mg of feces.[38–40] An optical density value >0.05 was considered positive according to the manufacturer's instructions.

Feces were evaluated for the presence of DNA and RNA from CPV2 and CCV by qPCR and qRT-PCR, respectively, as previously described.[6] Results from duplicate PCR analyses from the

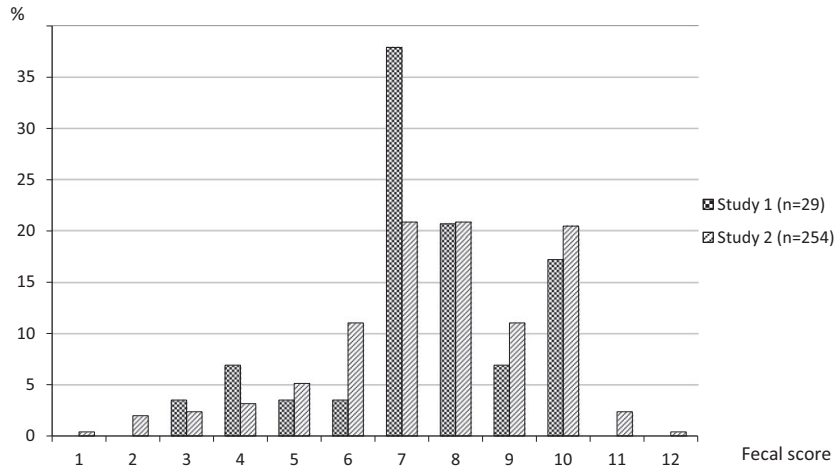

Fig 1. Fecal quality in puppies included in both studies.

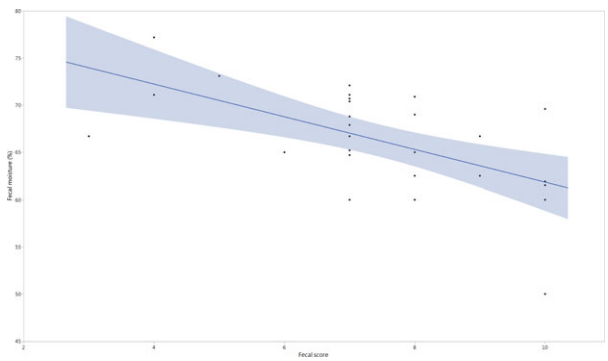

Fig 2. Correlation between fecal moisture and fecal score in 29 puppies. Scatter plot of the data overlaid with the regression line, and 95% confidence interval (gray zone). Each black point represents a dog ($r = -0.59$; $P = .001$).

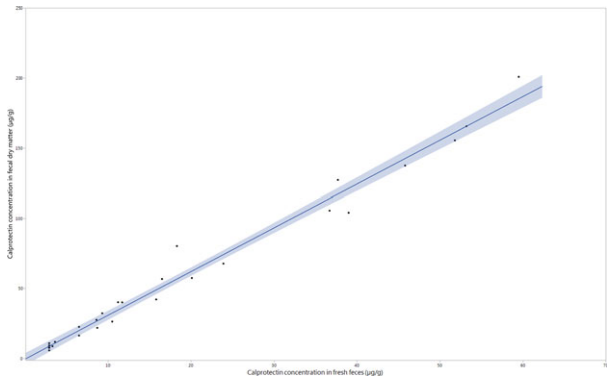

Fig 3. Correlation between fecal calprotectin concentration in fresh feces and fecal dry matter (n = 29). Scatter plot of the data overlaid with the regression line, and 95% confidence interval (grey zone). Each black point represents a dog ($r = 0.98$; $P < .001$).

extracted DNA (ie, 2 PCR assay were performed for each fecal extract) were expressed semiquantitatively as virus loads. Puppies were defined as infected by CPV2 and CCV if viral loads were $>10^{10.3}$ and $10^{9.3}$ copies respectively.[6]

After extraction, calprotectin and IgA were quantified by in-house radioimmunoassays as previously described.[22,32,34] All samples were analyzed using the same batch of tracer and reagents.

Data Management and Statistical Analysis

Data are shown as the median and range (min–max). Statistical analyses were performed using a commercial software package.[c]

Spearman's rho correlation coefficient was used to evaluate the correlation between fecal concentrations of each marker in fresh feces and fecal dry matter.

Number of puppies with positive and negative fecal test results for each enteropathogen was tabulated by age of the puppies. The significance of the univariate association between age and the shedding of each enteropathogen was determined using chi-squared-tests. A P value <.05 was considered statistically significant.

To assess the association between enteropathogens sheddings and fecal IgA and calprotectin concentrations in puppies, 4 statistical models were performed for each marker. In a first step, univariate analyses (Mann–Whitney and Kruskal–Wallis tests) were performed to evaluate a possible association of each factor on either fecal marker. Variables examined included age of puppies (5–6/7–8/9–11 weeks of age), breed size (small/large), fecal quality (normal/abnormal), *G. inestinalis, C. ohioensis complex, C. canis, Toxocara canis*, CPV2, and CCV shedding (yes/no), shedding of ≥1virus (yes/no), shedding of ≥1parasite (yes/no), and shedding of ≥1 enteropathogen (yes/no). In a second step, relationships between shedding of enteropathogens and fecal marker concentrations were evaluated in 3 different linear mixed models for each marker. In a first linear mixed model, effects of each pathogen (shedding of each pathogen [yes/no]) on either marker were evaluated. In a second linear mixed model, influence of the type of enteropathogens (shedding of ≥1 parasite [yes/no] and shedding of ≥1 virus [yes/no]) on either fecal marker was evaluated. In a last linear mixed model, global effect of enteropathogens (shedding of ≥1 enteropathogen [yes/no]) on both fecal markers was evaluated. In all of these mixed models, breed size and age of puppies were included as fixed effects and litter variable nested within breeding kennel was defined as a random term. For each model, the normality of residuals distribution was assessed using the Shapiro-Wilk test. According to residuals distribution for each of the multivariable models, the outcome was log transformed (fecal calprotectin concentration) or rank transformed

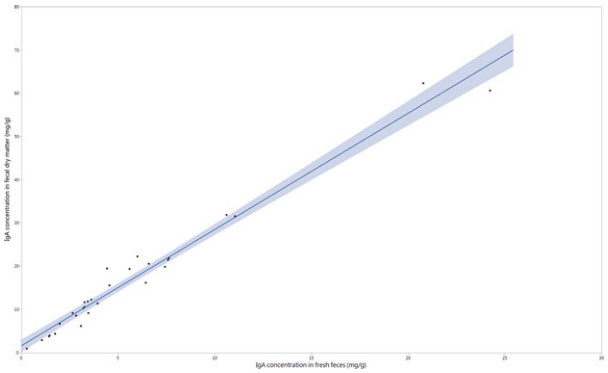

Fig 4. Correlation between fecal IgA concentration in fresh feces and fecal dry matter (n = 29). Scatter plot of the data overlaid with the regression line, and 95% confidence interval (grey zone). Each black point represents a dog (r = 0.97; P < .001).

(fecal IgA concentration). Differences were considered significant for P values <.05. Quantitative data are presented as medians with ranges.

Results

Study 1

Seventy puppies (64 classified as belonging to a large breed) were included in the study (mean age, 8.8 weeks; range 6–14 weeks). Among these, 29 (41%) puppies defecated a sufficient volume of feces. These puppies consisted of large breed puppies between 6 and 10 weeks of age (mean age, 8.5 weeks). A median fecal score of 7 was obtained (range, 3–10; Fig 1) in the 29 puppies that defecated a sufficient volume of feces. Fecal moisture ranged from 50 to 77.2% (median, 66.7%), with a strong negative correlation with fecal scores (r, −0.59; P = .001; Fig 2).

Fecal calprotectin concentrations in fresh feces ranged from 2.9 to 59.5 μg/g (median, 10.5 μg/g), and from 5.8 to 200.8 μg/g (median, 32.2 μg/g) in fecal dry matter. A strong positive correlation was observed between fecal calprotectin concentration both in fresh feces and in fecal dry matter (r, 0.98; P < .001; Fig 3). A moderate negative correlation was observed between fecal scores and fecal calprotectin concentrations in fresh feces and in fecal dry matter (r, −0.38; P = .045; and r, −0.49; P = .007 respectively).

Fecal IgA concentrations in fresh feces ranged from 0.3 to 24.2 mg/g (median, 3.6 mg/g) and from 0.9 to 62.3 mg/g (median, 11.8 mg/g) in fecal dry matter. A strong positive correlation was observed between fecal IgA concentrations in fresh feces and in fecal dry matter (r, 0.97; P < .001; Fig 4). A moderate negative correlation was observed between fecal scores and fecal IgA concentrations in either fresh feces or fecal dry matter (r, −0.53; P = .003 and r, −0.64; P < .001 respectively).

Study 2

Among the 254 puppies included in the study, 180 (71%) were large breed puppies. Puppies were between 5 and 11 weeks of age (mean, 7.7 weeks). The mean number of puppies included in each kennel was 8 (range, 1–18). A median fecal score of 8 was obtained (range, 1–12; Fig 1).

Table 1. Frequency of coshedding of enteropathogens in 254 puppies.

CPV2	CCV	*Toxocara canis*	*Cystoisospora ohioensis* Complex	*Cystoisospora canis*	*Giardia* Sp.
CPV2	3.9 (10)	3.5 (9)	7.1 (18)	6.7 (17)	9.4 (24)
	CCV	2.8 (7)	5.5 (14)	0.4 (1)	16.5 (42)
		T. canis	11.4 (29)	3.1 (8)	3.1 (8)
			C. ohioensis complex	1.6 (4)	4.3 (11)
				C. canis	8.7 (22)
					Giardia sp.

Data are shown as % (number) of puppies.

Table 2. Prevalence of enteropathogens shedding based on age (n = 254).

		Age of Puppies			
Pathogens	Total Prevalence (n = 254) % (n_i)	5–6 weeks (n = 40) % (n_i)	7–8 weeks (n = 141) % (n_i)	9–11 weeks (n = 73) % (n_i)	Global P-Value
Coronavirus	22 (56)	10 (4)[a]	19.1 (27)[a]	34.2 (25)[b]	.006
Parvovirus	18.9 (48)	22.5 (9)	19.9 (28)	15.1 (11)	.571
Giardia	37.8 (96)	25 (10)[a]	29.8 (42)[a]	60.3 (44)[b]	<.001
Toxocara canis	21.3 (54)	40 (16)[a]	21.3 (30)[b]	11 (8)[b]	.001
Cystoisospora ohioensis complex	30.3 (77)	32.5 (13)[a]	36.9 (52)[a]	16.4 (12)[b]	.008
Cystoisospora canis	10.6 (27)	25 (10)[a]	5 (7)[b]	13.7 (10)[a]	<.001

n_i = number of puppies infected for the category considered; n = total number of puppies in the category considered.
For each line, categories with different letters (a, b) were significantly different (P < .05).

In general, 2 different enteric viruses and 4 parasites were identified (Table 1). At least 1 enteropathogen was identified in 75.6% (192/254) of the puppies. 71.7% (182/254) of puppies were infected by ≥1 parasite and 37% (94/254) by ≥1 of the 2 viruses tested. One-third (84/254) of the puppies were infected simultaneously with ≥1 virus and 1 parasite (Table 2). Puppies between 5 and 8 weeks of age had a significantly higher prevalence of *C. ohioensis* complex and a lower prevalence of CCV and *G. duodenalis* than puppies between 9 and 11 weeks of age (Table 1).

Fecal calprotectin concentrations ranged from 2.9 to 421.4 μg/g feces (median, 15.2 μg/g feces). Of the 254 puppies included, 44 (17%) had a fecal concentration >49 μg/g feces (threshold of clinical interest).[19] Fecal calprotectin concentration was significantly affected by age ($P = .001$) but not by breed size ($P = .217$), viral infection (CPV2, CCV, or both ; $P = .863$), or parasitic infection (*G. duodenalis, C. ohioensis* complex, *C. canis*, *T. canis, or both*; $P = .791$; Table 3). Fecal calprotectin concentration was not associated with fecal score ($P = .851$). The concentration of fecal calprotectin was higher and more variable in younger puppies between 5 and 8 weeks of age than in the older puppies (9–11 weeks of age; Fig 5). Twenty-two, 21, and 7% of puppies had fecal calprotectin concentrations >49 μg/g feces at, respectively, 5–6, 7–8, and 9–11 weeks of age.

Fecal IgA concentration ranged from 0.1 to 27.2 mg/g feces (median, 4.5 mg/g feces). In contrast with calprotectin, IgA concentration was significantly influenced

Table 3. Evaluation of factors influencing fecal calprotectin concentrations in 254 puppies (univariate and multivariate analyses).

			Linear Mixed Model		
Variables	Fecal Calprotectin Concentration Median [range]	Initial Unadjusted Analysis (*P*-Value)	Each Pathogen Evaluated Individually (*P*-Value)	Shedding of at Least One Parasite or One Virus (*P*-Value)	Shedding of at Least One Pathogen (*P*-Value)
Age		**.001**			
5–6 weeks	17.8 [2.9–352.9]	–	–	–	–
7–8 weeks	18.8 [2.9–421.4]		.202	.203	.177
9–11 weeks	5.5 [2.9–69.3]		**.004**	**.002**	**.002**
Breed size					
Small	19.8 [2.9–222.5]	.096	.139	.218	.217
Large	11.9 [2.9–421.4]				
Fecal score					
Normal	14.6 [2.9–421.4]	.851	–	–	–
Abnormal	18.1 [2.9–127.3]				
Giardia					
No shedding	19.6 [2.9–421.4]	**.007**	.602	–	–
Shedding	6.5 [2.9–222.5]				
Cystoisospora ohioensis					
No shedding	9.9 [2.9–352.9]	.104	.056	–	–
Shedding	2.5 [2.9–421.4]				
Cystoisospora canis					
No shedding	15 [2.9–421.4]	.348	.73	–	–
Shedding	15.3 [2.9–352.9]				
Toxocara canis					
Not shedding	11.9 [2.9–352.9]	**.005**	.87	–	–
Shedding	3.4 [2.9–421.4]				
CPV2					
No shedding	14.2 [2.9–421.4]	.692	.65	–	–
Shedding	18 [2.9–352.9]				
CCV					
No shedding	17 [2.9–421.4]	.127	.67	–	–
Shedding	6.5 [2.9–123.8]				
Infection by at least one virus		.502	–	.863	–
No shedding	16.7 [2.9–421.4]				
Shedding	1.9 [2.9–352.9]				
Shedding of at least one parasite		.788	–	.791	–
No shedding	16.5 [2.9–93]				
Shedding	13.9 [2.9–421.4]				
Shedding of at least one enteropathogen		.92	–		.695
No shedding	16.5 [2.9–93]				
Shedding	13.9 [2.9–421.4]				

Bolded numbers are numbers with a *P*-value ≤.05.

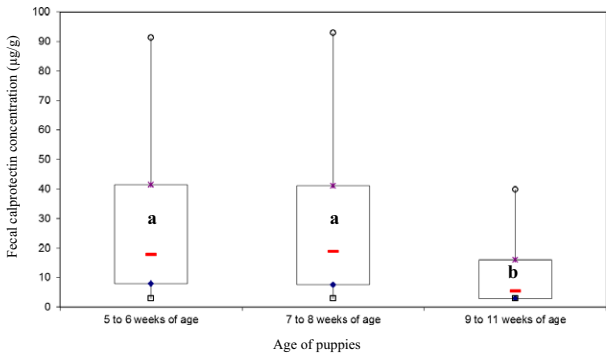

Fig 5. Box-and-whisker plots of fecal calprotectin concentrations in 254 puppies. Each box represents the first to the third quartiles (25th to 75th percentiles), the bar in each box represents the median, and the whiskers represent the first to ninth decile (10th to 90th percentiles). Values with different letters (a,b) differ significantly ($P < .05$).

by enteropathogen shedding ($P = .01$) but by none of the other factors tested (Table 4). Fecal IgA concentrations were 1.4 times lower in puppies that were shedding at least 1 enteropathogen (median, 4.1 mg/g feces; range, 0.1–22.7 mg/g feces) than in puppies without enteropathogen shedding (median, 5.7 mg/g feces; range, 0.5–27.2 mg/g feces; Fig 6). Fecal IgA concentration was not found to be associated with fecal score ($P = .891$).

Discussion

Diarrhea is common in puppies around the time of weaning, and may be accompanied by slowed growth of the puppies.[6] Viral and parasitic infections are very common in young puppies and are involved in weanling diarrhea.[5,6,14,41–43] The early detection of such infections would avoid growth retardation and could decrease the development of more severe forms of the disease. Thus, noninvasive markers of digestive health, the concentrations of which might be modified by the presence of enteropathogens, would be of great utility in these patients. Thus, our study investigated 2 fecal markers, calprotectin and IgA, used in human pediatric gastroenterology for their utility in weanling puppies. In our study, puppies that shed ≥1 enteropathogen had significantly lower fecal IgA concentrations than did puppies without any enteropathogen shedding identified. This lower fecal IgA concentration may be a cause or a consequence of the enteropathogen shedding. Immunoglobulin A can actively bind microrganisms, enterotoxins, and other antigens, and prevent adherence and subsequent penetration of the intestinal wall. Thus, a lower fecal IgA concentration could be caused by IgA being utilized in antigen binding or by enterohepatic recirculation of IgA. After IgA binds an antigen in the intestinal lumen, it is either excreted in the feces or is actively reabsorbed for destruction of the microorganism or virus by hepatic Kuppfer cells. Reabsorption of IgA could have been increased by an infection with an enteropathogen, which could result in decreased fecal concentrations of IgA. Conversely, the lower fecal IgA concentration also could indicate the presence of altered local immunity and thus serve as evidence for a higher risk of infection with an enteropathogen. A positive impact of fecal IgA on protection against infectious diseases already has been described in other species. Mice lacking secretory IgA exhibit a significant delay in clearance of rotavirus infection compared with mice that have secretory IgA.[44] In children, fecal IgA concentrations also were shown to have an influence on protection against rotavirus infection and resulting disease.[45]

No significant effect of any viral (CCV or CPV2) or parasite shedding (*G. duodenalis, C. ohioensis* complex, *C. canis*, or *T. canis)* on fecal calprotectin concentration was observed. This lack of difference in calprotectin concentrations between dogs that showed enteropathogen shedding and those that did not could be explained by the population of dogs enrolled in our study (ie, healthy puppies or puppies presenting only with an abnormal fecal quality without any other clinical sign). In humans, the patient's clinical status influences the concentrations of this marker. In children who are clinically healthy but infected by *Giardia*, no effect on fecal calprotectin concentration was described.[33] However, in human patients with viral gastroenteritis, fecal calprotectin concentrations were reported to be associated with the severity of clinical signs.[46] In our study, 18.9% of puppies were found to be excreting a high load of CPV2 but without any of the typical clinical signs (eg, hemorrhagic diarrhea, vomiting, prostration, dehydration, anorexia). This healthy carrier state could explain the lack of association between shedding of this virus and fecal calprotectin concentrations. Another study comparing fecal calprotectin concentrations among healthy puppies, puppies with an abnormal fecal quality, and puppies with clinical parvovirus infection would be needed to further elucidate this relationship.

Fecal moisture in our study ranged from 50 to 77.2% (median, 66.7%), with a negative correlation with fecal scores, which is accordance with previous studies.[10,30] A negative correlation also was observed between fecal markers and fecal score. The higher IgA and calprotectin concentrations in puppies with liquid or soft feces in this study do not seem to be a direct consequence of stool consistency (dilution) because this negative correlation was observed for fresh feces as well as for concentrations based on fecal dry matter. The negative correlation between fecal score and fecal marker concentration could be explained by the effect of age acting as a confounding factor. Indeed age influence feces quality (lower fecal score in very young puppies)[6] and, at the same time, age influences fecal concentrations of both markers (higher fecal concentrations in very young puppies).[47]

Our study indicates that fecal calprotectin concentrations decrease and stabilize with age. This result is in accordance with our longitudinal study performed in young dogs around the age of weaning.[47] In humans, considerably higher fecal calprotectin concentrations also have been observed in infants around the time of

Table 4. Evaluation of factors influencing fecal IgA concentrations in 254 puppies (univariate and multivariate analyses).

Variables	Fecal IgA Concentration Median [Range]	Initial Unadjusted Analysis (*P*-Value)	Linear Mixed Model		
			Each Pathogen Evaluated Individually (*P*-Value)	Shedding of at Least One Parasite or One Virus (*P*-Value)	Shedding of at Least One Pathogen (*P*-Value)
Age					
5–6 weeks	2.3 [0.3–22.7]	**.003**	–	–	–
7–8 weeks	5.5 [0.1–24.2]		.971	.506	.489
9–11 weeks	4.1 [0.2–27.2]		.375	.193	.176
Breed size					
Small	5.7 [0.14–19.2]	**.019**	.091	.138	.197
Large	3.9 [0.1–27.2]				
Fecal score					
Normal	4.4 [0.1–27.2]	.891	–	–	–
Abnormal	5.4 [0.3–21.9]				
Giardia					
No shedding	4.9 [0.1–27.2]	.816	.127	–	–
Shedding	4.3 [0.1–22.7]				
Cystoisospora ohioensis					
No shedding	4.5 [0.1–27.2]	.126	.333	–	–
Shedding	4.5 [0.1–20.8]				
Cystoisospora canis					
No shedding	4.4 [0.1–27.2]	**.048**	**.021**	–	–
Shedding	5.5 [0.2–22.7]				
Toxocara canis					
No shedding	4.8 [0.1–27.2]	.165	.415	–	–
Shedding	4 [0.1–20.8]				
CPV2					
No shedding	4.5 [0.1–27.2]	.535	.373	–	–
Shedding	3.8 [0.3–21.9]				
CCV					
No shedding	4.8 [0.1–27.2]	.118	.802	–	–
Shedding	3.3 [0.1–20.8]				
Infection by at least one virus		.743	–	.864	–
No shedding	4.8 [0.1–27.2]				
Shedding	3.7 [0.1–21.9]				
Infection by at least one parasite		.062	–	.058	–
No shedding	5.5 [0.5–27.2]				
Shedding	4.2 [0.1–22.7]				
Infection by at least one enteropathogen		.072	–	–	**.01**
No shedding	5.7 [0.5–27.2]				
Shedding	4.1 [0.1–22.7]				

Bolded numbers are numbers with a *P*-value ≤.05

birth compared with those in healthy older children and adults.[33,48–50] In our study, 17% of puppies had high fecal calprotectin concentrations (>49 μg/g) similar to those observed in adult dogs with inflammatory bowel disease, with large interindividual variations.[19] These high concentrations do not appear to be linked to viral or parasite shedding because this effect of age on fecal calprotectin concentrations was still observed when both variables (age and enteropathogen shedding) were taken into consideration within the same statistical model. Moreover, we previously observed a spontaneous normalization of fecal calprotectin concentrations in healthy puppies during the weaning period.[47] The type of food (eg, natural milk, industrial milk, dry food) may have influenced fecal calprotectin concentrations. Human infants who are exclusively breastfed show significantly higher fecal calprotectin concentrations compared to those receiving a mixed diet.[49,51] The effect of natural milk may depend on several factors such as hormones (eg, ghrelin, leptin), cytokines and other immunostimulants and growth factors (eg, epidermal growth factor, granulocyte colony-stimulating factor), which all contribute to the development of the gastrointestinal immune system.[51] Milk ingestion was not controlled in our study, with puppies having free access to maternal milk. However, from 5 to 8 weeks of age, the proportion of natural maternal milk decreases continuously in a puppie's diet because of physiologic progressive weaning. Developmental processes occurring in the digestive tract during this period

Fig 6. Box-and-whisker plots of fecal IgA concentrations in 254 puppies. Each box represents the first to the third quartiles (25th to 75th percentiles), the bar in each box represents the median, and the whiskers represent the first to ninth decile (10th–90th percentiles). Values with different letters (a,b) differ significantly ($P = .01$).

of life also could explain the higher fecal calprotectin concentrations. During the first weeks of life, intestinal permeability is higher[52], which may lead to transepithelial migration of neutrophils, as observed in adults with inflammatory bowel disease.[53] The physiological establishment and stabilization of the gut microbiota also may have an effect on calprotectin release as has been suggested in humans.[54,55] The higher calprotectin concentrations observed also could be linked to bacterial gastrointestinal infections as described in children.[15,56]

Conclusion

Our study indicates that fecal calprotectin and IgA are of no diagnostic value to detect the presence of an enteropathogen in clinically healthy puppies or puppies with abnormal feces. However, these markers might be useful to better understand the maturation of the digestive tract, the development of systemic and local immunity, and the establishment and stabilization of the gut microbiota. The development of noninvasive fecal biomarkers that may prove to be useful to evaluate gastrointestinal health in puppies remains a challenge.

Footnotes

[a] Copan, Brescia, Italy
[b] ProSpecT-Giardia Microplate Assay kit, Remel, France
[c] SAS, version 9.3, SAS Institute Inc., Cary, NC

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Conflict of Interest Declaration: No product branded by Royal Canin was tested in the experiment and authors belonging to the Royal Canin staff have no conflict of interest to declare. Other authors also declare no conflict of interest.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

References

1. Freeman LM, Abood SK, Fascetti AJ, et al. Disease prevalence among dogs and cats in the United States and Australia and proportions of dogs and cats that receive therapeutic diets or dietary supplements. J Am Vet Med Assoc 2006;229:531–534.

2. Hubbard K, Skelly BJ, McKelvie J, et al. Risk of vomiting and diarrhoea in dogs. Vet Rec 2007;161:755–757.

3. Jones PH, Dawson S, Gaskell RM, et al. Surveillance of diarrhoea in small animal practice through the Small Animal Veterinary Surveillance Network (SAVSNET). Vet J 2014;201:412–418.

4. Tupler T, Levy JK, Sabshin SJ, et al. Enteropathogens identified in dogs entering a Florida animal shelter with normal feces or diarrhea. J Am Vet Med Assoc 2012;241:338–343.

5. Grellet A, Chastant-Maillard S, Robin C, et al. Risk factors of weaning diarrhea in puppies housed in breeding kennels. Prev Vet Med 2014;117:260–265.

6. Grellet A, Feugier A, Chastant-Maillard S, et al. Validation of a fecal scoring scale in puppies during the weaning period. Prev Vet Med 2012;106:315–323.

7. Weber MP, Stambouli F, Martin LJ, et al. Influence of age and body size on gastrointestinal transit time of radiopaque markers in healthy dogs. Am J Vet Res 2002;63:677–682.

8. Sokolow SH, Rand C, Marks SL, et al. Epidemiologic evaluation of diarrhea in dogs in an animal shelter. Am J Vet Res 2005;66:1018–1024.

9. Stavisky J, Radford AD, Gaskell R, et al. A case-control study of pathogen and lifestyle risk factors for diarrhoea in dogs. Prev Vet Med 2011;99:185–192.

10. Weber M, Martin L, Biourge V, et al. Influence of age and body size on the digestibility of a dry expanded diet in dogs. J Anim Physiol Anim Nutr (Berl) 2003;87:21–31.

11. Hernot DC, Dumon HJ, Biourge VC, et al. Evaluation of association between body size and large intestinal transit time in healthy dogs. Am J Vet Res 2006;67:342–347.

12. Hackett T, Lappin MR. Prevalence of enteric pathogens in dogs of North-central Colorado. J Am Anim Hosp Assoc 2003;39:52–56.

13. Buehl IE, Prosl H, Mundt HC, et al. Canine isosporosis—epidemiology of field and experimental infections. J Vet Med B Infect Dis Vet Public Health 2006;53:482–487.

14. Epe C, Rehkter G, Schnieder T, et al. Giardia in symptomatic dogs and cats in Europe—results of a European study. Vet Parasitol 2010;173:32–38.

15. Sykora J, Siala K, Huml M, et al. Evaluation of faecal calprotectin as a valuable non-invasive marker in distinguishing gut pathogens in young children with acute gastroenteritis. Acta Paediatr 2010;99:1389–1395.

16. Tibble J, Teahon K, Thjodleifsson B, et al. A simple method for assessing intestinal inflammation in Crohn's disease. Gut 2000;47:506–513.

17. Carroll D, Corfield A, Spicer R, et al. Faecal calprotectin concentrations and diagnosis of necrotising enterocolitis. Lancet 2003;361:310–311.

18. Bunn SK, Bisset WM, Main MJ, et al. Fecal calprotectin as a measure of disease activity in childhood inflammatory bowel disease. J Pediatr Gastroenterol Nutr 2001;32:171–177.

19. Grellet A, Heilmann RM, Lecoindre P, et al. Fecal calprotectin concentrations in adult dogs with chronic diarrhea. Am J Vet Res 2013;74:706–711.

20. Heilmann RM, Jergens AE, Ackermann MR, et al. Serum calprotectin concentrations in dogs with idiopathic inflammatory bowel disease. Am J Vet Res 2012;73:1900–1907.

21. Albers R, Antoine JM, Bourdet-Sicard R, et al. Markers to measure immunomodulation in human nutrition intervention studies. Br J Nutr 2005;94:452–481.

22. Tress U, Suchodolski JS, Williams DA, et al. Development of a fecal sample collection strategy for extraction and quantification of fecal immunoglobulin A in dogs. Am J Vet Res 2006;67: 1756–1759.

23. Peters IR, Calvert EL, Hall EJ, et al. Measurement of immunoglobulin concentrations in the feces of healthy dogs. Clin Diagn Lab Immunol 2004;11:841–848.

24. Weber M, Stambouli F, Martin L, et al. Gastrointestinal transit of solid radiopaque markers in large and giant breed growing dogs. J Anim Physiol Anim Nutr (Berl) 2001;85:242–250.

25. Kirkwood J. The influence of size on the biology of the dog. J Small Anim Pract 1985;26:97–110.

26. Meyer H, Kienzle E, Zentek J. Body size and relative weights of gastrointestinal tract and liver in dogs. J Vet Nutr 1993;2:31–35.

27. Herschel DA, Argenzio RA, Southworth M, et al. Absorption of volatile fatty acid, Na, and H2O by the colon of the dog. Am J Vet Res 1981;42:1118–1124.

28. Meyer H, Zentek J, Habernoll H, et al. Digestibility and compatibility of mixed diets and faecal consistency in different breeds of dog. Zentralbl Veterinarmed A 1999;46:155–165.

29. Rolfe VE, Adams CA, Butterwick RE, et al. Relationships between fecal consistency and colonic microstructure and absorptive function in dogs with and without nonspecific dietary sensitivity. Am J Vet Res 2002;63:617–622.

30. Weber MP, Hernot D, Nguyen PG, et al. Effect of size on electrolyte apparent absorption rates and fermentative activity in dogs. J Anim Physiol Anim Nutr (Berl) 2004;88:356–365.

31. Zaine L, Ferreira C, Gomes Mde O, et al. Faecal IgA concentration is influenced by age in dogs. Br J Nutr 2011;106(Suppl 1):S183–S186.

32. Grellet A, Mila H, Heilmann RM, et al. Effect of age, gestation and lactation on faecal IgA and calprotectin concentrations in dogs. J Nutr Sci 2014;3:1–5.

33. Hestvik E, Tumwine JK, Tylleskar T, et al. Faecal calprotectin concentrations in apparently healthy children aged 0-12 years in urban Kampala, Uganda: A community-based survey. BMC Pediatr 2011;11:9.

34. Heilmann RM, Suchodolski JS, Steiner JM. Development and analytic validation of a radioimmunoassay for the quantification of canine calprotectin in serum and feces from dogs. Am J Vet Res 2008;69:845–853.

35. Bauer BU, Pomroy WE, Gueydon J, et al. Comparison of the FLOTAC technique with the McMaster method and the Baermann technique to determine counts of Dictyocaulus eckerti L1 and strongylid eggs in faeces of red deer (Cervus elaphus). Parasitol Res 2010;107:555–560.

36. Baek BK, Kim CS, Kim JH, et al. Studies on isosporosis in dogs. I: Isolation and sporulation of Isospora ohioensis. Korean J Parasitol 1993;31:201–206.

37. Levine ND, Ivens V. Isospora species in the dog. J Parasitol 1965;51:859–864.

38. Decock C, Cadiergues MC, Larcher M, et al. Comparison of two techniques for diagnosis of giardiasis in dogs. Parasite 2003;10:69–72.

39. Rimhanen-Finne R, Enemark HL, Kolehmainen J, et al. Evaluation of immunofluorescence microscopy and enzyme-linked immunosorbent assay in detection of Cryptosporidium and Giardia infections in asymptomatic dogs. Vet Parasitol 2007;145:345–348.

40. Mekaru SR, Marks SL, Felley AJ, et al. Comparison of direct immunofluorescence, immunoassays, and fecal flotation for detection of *Cryptosporidium* spp. and *Giardia* spp. in naturally exposed cats in 4 Northern California animal shelters. J Vet Intern Med 2007;21:959–965.

41. Gates MC, Nolan TJ. Endoparasite prevalence and recurrence across different age groups of dogs and cats. Vet Parasitol 2009;166:153–158.

42. Gates MC, Nolan TJ. Risk factors for endoparasitism in dogs: Retrospective case-control study of 6578 veterinary teaching hospital cases. J Small Anim Pract 2009;50:636–640.

43. Sakulwira K, Vanapongtipagorn P, Theamboonlers A, et al. Prevalence of canine coronavirus and parvovirus infections in dogs with gastroenteritis in Thailand. Vet Med – Czech 2003;48:163–167.

44. Blutt SE, Miller AD, Salmon SL, et al. IgA is important for clearance and critical for protection from rotavirus infection. Mucosal Immunol 2012;5:712–719.

45. Coulson BS, Grimwood K, Hudson IL, et al. Role of coproantibody in clinical protection of children during reinfection with rotavirus. J Clin Microbiol 1992;30:1678–1684.

46. Chen CC, Huang JL, Chang CJ, et al. Fecal calprotectin as a correlative marker in clinical severity of infectious diarrhea and usefulness in evaluating bacterial or viral pathogens in children. J Pediatr Gastroenterol Nutr 2012;55:541–547.

47. Grellet A, Mila H, Heilmann RM, et al. Effect of age, gestation and lactation on faecal IgA and calprotectin concentrations in dogs. J Nutr Sci 2014;3:e41.

48. Konikoff MR, Denson LA. Role of fecal calprotectin as a biomarker of intestinal inflammation in inflammatory bowel disease. Inflamm Bowel Dis 2006;12:524–534.

49. Dorosko SM, Mackenzie T, Connor RI. Fecal calprotectin concentrations are higher in exclusively breastfed infants compared to those who are mixed-fed. Breastfeed Med 2008;3: 117–119.

50. Rouge C, Butel MJ, Piloquet H, et al. Fecal calprotectin excretion in preterm infants during the neonatal period. PLoS One 2010;5:e11083.

51. Savino F, Castagno E, Calabrese R, et al. High faecal calprotectin levels in healthy, exclusively breast-fed infants. Neonatology 2010;97:299–304.

52. Weber MP, Martin LJ, Dumon HJ, et al. Influence of age and body size on intestinal permeability and absorption in healthy dogs. Am J Vet Res 2002;63:1323–1328.

53. Berstad A, Arslan G, Folvik G. Relationship between intestinal permeability and calprotectin concentration in gut lavage fluid. Scand J Gastroenterol 2000;35:64–69.

54. Baldassarre ME, Altomare MA, Fanelli M, et al. Does calprotectin represent a regulatory factor in host defense or a drug target in inflammatory disease? Endocr Metab Immune Disord Drug Targets 2007;7:1–5.

55. Josefsson S, Bunn SK, Domellof M. Fecal calprotectin in very low birth weight infants. J Pediatr Gastroenterol Nutr 2007;44:407–413.

56. Shastri YM, Bergis D, Povse N, et al. Prospective multicenter study evaluating fecal calprotectin in adult acute bacterial diarrhea. Am J Med 2008;121:1099–1106.